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1. Dash et al, Molecular Brain Research 39(1-2) 1996 43-51

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5. Kaang et al Neuron March 1993 10(3) pages 427-35

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9. Brockeroff et al FASEB J 4(4) 1990 A899

10. Thank you!

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Protein synthesis during acquisition of long-term facilitation is needed for the persistent loss of regulatory subunits of the *Aplysia* cAMP-dependent protein kinase

(cAMP/protein kinase regulation/sensitization/learning/memory)

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ABSTRACT **Depending on the number or the length of exposure, application of serotonin can produce either short-term or long-term presynaptic facilitation of *Aplysia* sensory-to-motor synapses. The cAMP-dependent protein kinase, a heterodimer of two regulatory and two catalytic subunits, has been shown to become stably activated only during long-term facilitation. Both acquisition of long-term facilitation and persistent activation of the kinase is blocked by anisomycin, an effective, reversible, and specific inhibitor of protein synthesis in *Aplysia*. We report here that 2-hr exposure of pleural sensory cells to serotonin lowers the concentration of regulatory subunits but does not change the concentration of catalytic subunits, as assayed 24 hr later; 5-min exposure to serotonin has no effect on either type of subunit. Increasing intracellular cAMP with a permeable analog of cAMP together with the phosphodiesterase inhibitor isobutyl methylxanthine also decreased regulatory subunits, suggesting that cAMP is the second messenger mediating serotonin action. Anisomycin blocked the loss of regulatory subunits only when applied with serotonin; application after the 2-hr treatment with serotonin had no effect. In the *Aplysia* accessory radula contractor muscle, prolonged exposure to serotonin or to the peptide transmitter small cardioactive peptide B, both of which produce large increases in intracellular cAMP, does not decrease regulatory subunits. This mechanism of regulating the cAMP-dependent protein kinase therefore may be specific to the nervous system. We conclude that during long-term facilitation, new protein is synthesized in response to the facilitatory stimulus, which changes the ratio of subunits of the cAMP-dependent protein kinase. This alteration in ratio could persistently activate the kinase and produce the persistent phosphorylation seen in long-term facilitated sensory cells.**

Sensitization of defensive reflexes in the marine mollusk *Aplysia* and the underlying presynaptic facilitation of sensory-to-motor neuron synapses can be short (lasting minutes) or long (lasting days to weeks) (1–3). During acquisition of the long-term process, serotonin, a facilitating transmitter, induces a persistent increase in cAMP-dependent protein phosphorylation in sensory neurons (4). The *Aplysia* cAMP-dependent protein kinase (A-kinase), a heterodimer of two regulatory subunits that inhibit the two catalytic subunits, is activated in both short-term (1, 4) and long-term facilitation (4) of both the gill- and siphon-withdrawal reflex and the tail-withdrawal reflex. The amount of regulatory subunits has been found to be decreased as compared with catalytic subunits in abdominal sensory cells of animals trained for long-term sensitization of the gill- and siphon-withdrawal reflex (5). These changes in regulatory subunits could account for the persistent protein phosphorylation seen in long-term facilitation (4). Evidence has been presented that long-term facilitation (6, 7), behavioral sensitization (8), and persistent phosphorylation (4) require synthesis of new proteins during a critical acquisition period. We examined the A-kinase in isolated clusters of pleural sensory cell bodies to determine whether facilitatory stimuli produce changes similar to those found in the intact animal (5) and whether these changes depend on the synthesis of new protein.

MATERIALS AND METHODS

Pleural–pedal ganglia were removed, and the pleural sensory cell body clusters were dissected free from anesthetized *Aplysia californica* (100–200 g) supplied by the Howard Hughes Medical Institute Mariculture Resource Facility (Woods Hole Oceanographic Institution, Woods Hole, MA) (9, 10). The paired accessory radula closer muscles also were isolated, as described by Weiss *et al.* (11). A pair of sensory clusters or muscles from a single animal was used for each determination. Right and left clusters were taken at random for the experimental and control groups. Each cluster was incubated in an artificial seawater supplemented with amino acids, sugars, vitamins, and antibiotics (12) at room temperature. After the experimental treatment, the clusters were washed extensively and maintained in the seawater for an additional 24 hr. They were then frozen in liquid nitrogen, rapidly thawed, homogenized in plastic tissue grinders (Kontes), and quantitatively photolabeled by the method of Greenberg *et al.* (5) with some modifications: 0.2 μCi of [^{32}P]8-azido-cAMP (ICN; 60–70 Ci/mmol; 1 Ci = 37 GBq) and 25 μl of SDS sample buffer were added; the mixture was then heated at 95°C for 5 min and electrophoresed on SDS/8% polyacrylamide gels (13). The gels were silver-stained, dried, and autoradiographed. The amounts of radioactivity in the M_r 47,000 and 52,000 cAMP-binding components in the sensory clusters or the M_r 55,000 component in muscle were determined by scanning the dried gels on an Ambis model radioisotope scanning system II (Ambis, San Diego, CA). The radioactivity in the M_r 105,000 binding component was too low for accurate measurement. Catalytic activity was assayed (14) with some modifications: 20 μCi of [γ-^{32}P]ATP (New England Nuclear) was added in the presence or absence of 5 μM A-kinase inhibitor from rabbit muscle (Walsh

Abbreviations: A-kinase, cAMP-dependent protein kinase; IBMX, isobutyl methylxanthine; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; SCP_B, small cardioactive peptide B.

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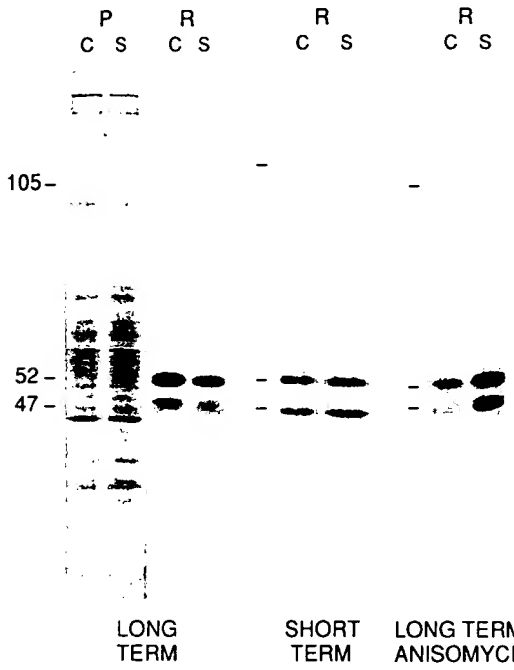

FIG. 1. Persistent decrease of regulatory subunits of the cAMP-dependent protein kinase in sensory cell clusters is induced by long-term exposure to serotonin and depends on synthesis of new protein. Paired control (C) or sensitized (S) pleural sensory cell clusters were treated for either 2 hr with serotonin (40 μM) (long term), 5 min with serotonin (short term), or preincubated for 30 min with anisomycin (10 μM) followed by 2 hr with serotonin together with anisomycin (long term + anisomycin). After 24 hr, clusters were homogenized, photoaffinity-labeled with [^{32}P]8-azido-cAMP, electrophoresed on polyacrylamide gels, silver stained (P), and then autoradiographed (R). M_r markers at left were multiplied by 10^{-3}.

inhibitor; Sigma) or 5 μM cAMP together with 100 μM isobutyl methylxanthine (IBMX).

RESULTS

Changes in the A-Kinase Induced by Prolonged Exposure to Serotonin. To produce either short-term or long-term facilitation, we applied serotonin to one of the pair of sensory cell clusters dissected from the bilateral pleural ganglia (4). The contralateral cluster from the same animal served as control (4, 10). We maintained the clusters in culture for 24 hr, extracted the proteins, and labeled them quantitatively with the photoaffinity reagent [^{32}P]8-azido-cAMP (5). In these extracts of *Aplysia* neurons, we observe specific labeling of three components with M_r values of 105,000, 52,000, and 47,000 (5, 14, 15) (Fig. 1). With a 2-hr application of serotonin, a treatment that results in long-term facilitation and in persistently increased protein phosphorylation (4), regulatory subunits were significantly decreased 24 hr after the serotonin treatment. In contrast, there was no change in total catalytic subunit activity (Table 1). When clusters were exposed to serotonin for 5 min, a treatment resulting only in short-term facilitation, no changes were seen in either subunit (Figs. 1 and 2; Table 1). As previously observed in the behavioral experiments with sensitized animals (5) loss of regulatory subunits is specific and does not result from general reduction of proteins in the treated cells: the intensity and pattern of silver-stained proteins in gel lanes containing experimental and control extracts are indistinguishable (Fig. 1). The isolated sensory clusters provide a more convenient experimental preparation for studying these changes than does the intact animal.

Loss of Regulatory Subunits Depends on Protein Synthesis During Exposure to Serotonin. Because acquisition of long-term synaptic facilitation by isolated sensory neurons (6, 7) and long-term behavioral sensitization (8) is blocked by inhibitors of protein synthesis, we tested whether the decrease of regulatory subunits seen 1 day after the 2-hr treatment with serotonin is similarly dependent for its induction on synthesis of new protein. We found that anisomycin, a specific, effective, and reversible inhibitor of protein synthesis in *Aplysia* (5, 16), blocked the loss of regulatory subunits produced by serotonin exposure. On the contrary, with serotonin, anisomycin not only blocked the reduction in regulatory subunits but also significantly increased the M_r 47,000 cAMP-binding component. Application of anisomycin alone had no effect (Fig. 2, Table 1).

Protein synthesis is required for long-term memory in vertebrates (17, 18), for the acquisition of long-term facilitation (6, 7), and for behavioral sensitization in *Aplysia* (8). To

Table 1. Effect of facilitatory treatments on amounts of A-kinase subunits

Treatment	Regulatory subunit, % control		Catalytic subunit, % control
	M_r 55,000	M_r 52,000 / M_r 47,000	
Pleural sensory cell cluster			
5-HT (2 hr)		66.3* ± 6.8 (12) / 66.1* ± 6.8 (12)	112.0 ± 11.0 (6)
5-HT (5 min)		105.5 ± 12.9 (9) / 104.5 ± 10.9 (9)	93.0 ± 12.6 (8)
5-HT + anisomycin (2 hr)		122 ± 11.2 (8) / 135† ± 14.8 (8)	
5-HT (2 hr) followed by anisomycin (3 hr)		65.5* ± 9.5 (8) / 65.5* ± 9.2 (8)	
Anisomycin (2 hr)		91.9 ± 11.5 (9) / 84.8 ± 11.5 (9)	
CTP-cAMP, IBMX (2 hr)		44.7‡ ± 6.6 (4) / 56.7‡ ± 4.9 (4)	
CTP-cAMP (2 hr)		102.2 ± 17.3 (7) / 104.5 ± 13.6 (7)	
IBMX (2 hr)		88.0 ± 5.8 (4) / 81.7 ± 6.7 (4)	
Muscle			
5-HT (1.5 hr)	97.0 ± 6.4 (4)		
5-HT (1.5 hr), immediate assay	103.9 ± 16.6 (4)		
SCP_B (1.5 hr)	96.3 ± 7.7 (3)		
SCP_B (1.5 hr), immediate assay	109.6 ± 2.2 (4)		

Regulatory and catalytic subunits of A-kinase were assayed in homogenates of pleural sensory cell clusters obtained from a single specimen. Regulatory subunits of accessory radula closure muscles from a single animal were assayed similarly. Values (mean ± SEM) were normalized by setting the control value to 100. The control value for incorporation of the photoaffinity reagent was 23.7 pmol/mg of protein ± 2.1, (n = 30). Number of experiments for each treatment is shown in parentheses. The control value for catalytic activity was 9.83 nmol/min per mg of protein ± 0.98, (n = 6). Values that differ significantly ($P < 0.05$, Student's t test) from control values were obtained after 5-min treatments with serotonin (5-HT) (*), 2-hr treatments with serotonin plus anisomycin (†), or 2-hr treatments with CPT-cAMP and IBMX (‡).

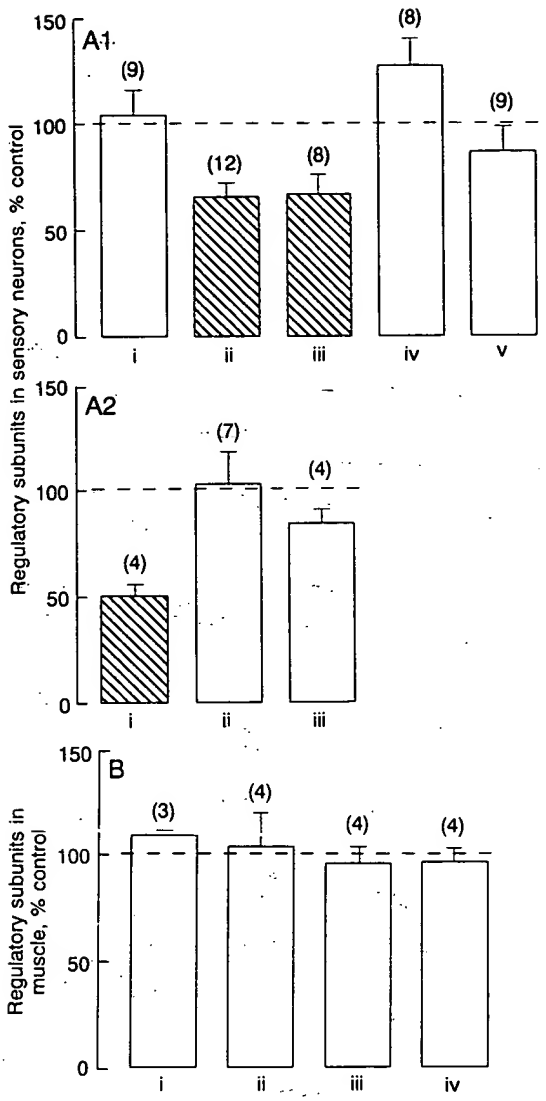

FIG. 2. Change in the amount of regulatory subunits in sensory clusters 24 hr after induction of either short-term or long-term facilitation by serotonin and by increased intracellular cAMP. Percent change (mean ± SEM) in regulatory subunits is determined by averaging the difference between the incorporation of [^{32}P]8-azido-cAMP in control and experimental clusters for M_r 52,000 and 47,000 components 24 hr after beginning each treatment. The change in the muscle M_r 55,000 regulatory subunit was assayed similarly. The muscle regulatory subunit of M_r 55,000 is structurally related to the nervous system subunits in the region of cAMP binding (7, 12). The number of times each experiment was done is shown in parentheses. Treatments that induce long-term facilitation are indicated by hatching. (*A1*) Decrease in regulatory subunits after treatments with serotonin that induce long-term facilitation has a critical period for new protein synthesis. The percent changes in regulatory subunits was determined 24 hr after treatment for 5 min with serotonin (40 μM) (bar i); 2 hr with serotonin (bar ii); 2 hr with serotonin followed by washing and 3 hr with anisomycin (10 μg/ml) (bar iii); with anisomycin added 30 min before serotonin, after which both serotonin and anisomycin were removed 2 hr later by washing extensively (bar iv); or 3 hr with anisomycin alone (bar v). (*A2*) Elevation of intracellular cAMP can produce the decrease in regulatory subunits. The change in regulatory subunits was determined 24 hr after a 2-hr treatment with CPT-cAMP (100 μM) with IBMX (100 μM) (bar i); CPT-cAMP (bar ii); or IBMX alone (bar iii). (*B*) Elevation of intracellular cAMP by serotonin (10 μM) or SCP_B (10 μM) has no effect on the muscle regulatory subunit. The change in regulatory subunits was determined immediately after (bars i and ii) or 24 hr after (bars iii and iv) 1.5-hr treatment with either SCP_B (bars i and iii) or serotonin (bars ii and iv).

block acquisition of long-term facilitation in *Aplysia*, however, protein synthesis need only be inhibited during the training period, which in our experiments would be equivalent to the period of serotonin exposure (6, 10). We find a similar critical period for the stable reduction of regulatory subunits. When anisomycin is applied after the 2-hr treatment with serotonin, it fails to block the decrease in regulatory subunits determined 24 hr later (Fig. 2; Table 1).

cAMP Can Mediate the Loss of Regulatory Subunits by Serotonin. Long-term facilitation can be produced either by increasing intracellular cAMP, by intracellular injection of cAMP (19, 20), or by exposure to a permeable analog together with a phosphodiesterase inhibitor (7). We also found that elevation of intracellular cAMP persistently decreases regulatory subunits in isolated sensory cell clusters. We applied either 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), a permeable analog of cAMP, or the phosphodiesterase inhibitor isobutyl methylxanthine (IBMX), or both together. No significant effect was seen with CPT-cAMP or IBMX alone, but application of them together decreases regulatory subunits (Fig. 2, Table 1). Persistent reduction of regulatory subunits is thus produced by two treatments that result both in long-term facilitation and persistent protein phosphorylation (application of serotonin for 2 hr or of CPT-cAMP with IBMX) and is not affected by four treatments that do not induce long-term facilitation (5 min with serotonin, 2 hr with serotonin in the presence of anisomycin, and treatment with either CPT-cAMP or IBMX alone) (4, 6–8).

Loss of Regulatory Subunits May Be Specific to Neurons Because It Does Not Occur in Muscle. Is this reduction in regulatory subunits a general feature of all cells that show a ligand-mediated increase in cAMP, or is it specific to nerve cells? To address this question we treated the biiateral accessory radula closer muscles of the *Aplysia* buccal mass with serotonin or small cardioactive peptide B (SCP_B). At the concentration used, both of these transmitters raise the amount of cAMP greater than 100-fold, increasing contractility of the muscles (21). This elevation of cAMP in buccal muscle is at least 25-fold greater than that in sensory neurons (22, 23). Despite the large cAMP increase, serotonin or SCP_B did not cause any change in regulatory subunits of the muscle A-kinase (Fig. 2*B*) either immediately after the treatment or 24 hr later, suggesting that regulation of R is specific to nerve cells.

DISCUSSION

Serotonin activates several enzymes responsible for signal transduction in *Aplysia* pleural sensory neurons, including the cAMP-dependent protein kinase (4, 9, 14, 20), protein kinase C (24, 25), and the Ca^{2+}/calmodulin protein kinase (26). The change in regulatory subunits produced by increased intracellular cAMP and the specific temporal requirement for new protein synthesis strongly suggest that the reduction of regulatory subunits plays a causal role in the persistent phosphorylation that accompanies long-term facilitation (4). Since increasing cAMP is sufficient to cause a persistent decrease in regulatory subunits, activation of A-kinase is likely to be responsible for the loss of regulatory subunits. Loss of regulatory subunits may result from a change in transcription or increased turnover (27–33). Alternatively, inhibition of protein synthesis might result in the disappearance of a labile regulator of regulatory subunits.

Regulatory subunit is known to be stabilized by association with catalytic subunit (27–29). Our results, however, suggest another mechanism for regulating the A-kinase. During acquisition of long-term facilitation we find that the amount of regulatory subunit in sensory neurons is regulated independently from the amount of catalytic subunit. In sensory neurons and, perhaps, in other neurons as well, there are

three distinct ways of regulating regulatory subunits, each of which gives rise to a kinase active for various periods of time. (*i*) Transient increases in cAMP, produced by serotonin and other transmitters, lead to the binding of cAMP to regulatory subunits, causing regulatory subunits to dissociate and activating catalytic subunits for as long as cAMP is elevated—usually for a period of minutes (9, 20, 22). (*ii*) Prolonged elevation of cAMP in nerve terminals leads to turnover of regulatory subunits by posttranslational mechanisms in the absence of new protein synthesis (2, 9, 32). This mechanism seems likely to operate in an intermediate time range of minutes to hours. We describe here a way of regulating regulatory subunits that appears specific to nerve cells. Repeated exposure of sensory cell bodies to serotonin or cAMP leads to the synthesis of new protein that stabilizes the reduction of regulatory subunits for at least 24 hr.

Two biochemical features now have emerged in the behavioral and cellular study of long-term memory: (*i*) a requirement for protein synthesis during acquisition of memory (3, 6, 17, 18) and (*ii*) a change of protein kinase activity in neurons important for memory. Persistent kinase activity has been demonstrated in long-term sensitization in *Aplysia* (4), conditioning of photoreceptor responses in *Hermisenda* (34), and in long-term potentiation in the vertebrate hippocampus (35). The evidence we present here suggests that these two features are causally related in *Aplysia* and constitute a long-term mechanism for regulating kinase activity. As long-term memory in other animals seems also to involve protein synthesis and persistent kinase activity, other protein kinases (for example protein kinase C and Ca^{2+}/calmodulin protein kinase II), also may be capable of being persistently modified as a consequence of altered gene expression. Thus, transcriptionally or translationally dependent persistence of kinase activity may be a general mechanism for the long-term synaptic plasticity important for memory.

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